RESEARCH REPORT
SWINE HEALTH

Title: PRRSV infection of pig macrophages - **NPB #06-130**

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Stated Objectives: The objective of this proposal is to transfer our recent studies on PRRSV infection of MARC-145 cells (available at *www.virologyj.com/content/3/1/90*) to studies of primary pig alveolar macrophages. The specific objectives are to investigate cell-to-cell PRRSV transmission and the response to antiviral agents, basically using this small grant to pave the way for future studies. Of particular interest are the viral dynamics and spread in vitro, as well as sensitivity to antiviral agents.

II. Industry Summary:

A. **Objectives.** The objective of this small grant were to was to investigate the regulation of PRRSV infection in cultured pig macrophages. The purpose of the work was to learn more about how PRRSV infects and spreads through cultured cells, and also to study the effects of antiviral agents on the virus. The results should help in the design of future PRRS treatments.

B. **Methods.** The research was conducted utilizing cells obtained from pig lungs (also known as porcine alveolar macrophages or PAMs). These cells were exposed to PRRSV under appropriate laboratory conditions, and the course of PRRSV infection was studied by a sensitive microscopy technique. Different antiviral treatments were applied to the cells in order to determine antiviral strategies which might work in pigs.

C. **Research findings.** From our research, PRRSV was found to replicate efficiently in some but not all PAMs, suggesting the physiological state of the target cells may regulate resistance to the virus. Consistent with this possibility, the course of infection was dramatically different in PAMs compared to that in another laboratory cell line (MARC-145 cells), since PRRSV was automatically down-regulated in PAMs after a relatively short period in culture. PRRSV infection of PAMS was found to be highly susceptible to antiviral treatment with two particular classes of drugs (proteins called cytokines which activate pig cell resistance to virus infection; and antibiotic molecules called quinolones). The two classes of drugs synergized to enhance pig cell resistance to PRRSV infection. These results are all consistent with the ability of pig macrophages to elaborate a natural resistance to PRRSV infection, which can also be induced by drug treatment. The funded research was included in a peer-reviewed publication, one national presentation, and one invention disclosure report.

D. **Significance for industry.** The results of this project describe a potentially important approach to prevention and/or treatment of PRRSV infection, based on regulation of cellular permissiveness to the virus. Drug synergy between antiviral cytokines and quinolones suggests a specific direction for the development of drug therapy for PRRS which might have acceptable toxicity (i.e. reduced side-effects) in pigs. Future research based on this pilot project could conceivably lead to effective preventive or therapeutic drugs for PRRS, as well as expand our knowledge of PRRSV regulation.

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III. Scientific Abstract:
With the assistance of funding from this small grant, PRRSV replication was studied in primary porcine alveolar macrophages (PAMs), under varying experimental conditions and in comparison with PRRSV infection of the standard MARC-145 cells used in many previous studies. Key findings to emerge were: 1. PRRSV replication in PAMs followed a distinct time-course, with limited spread and recovery in many cell cultures within 2 days post-infection. The PAM response to PRRSV is quite different from the cytopathic MARC-145 cell response to PRRSV infection; 2. PRRSV infection of PAMs was highly sensitive to combination low-dose treatment with antiviral cytokines (IFN-gamma or AK-2) and antiviral quinolone drugs. Consistent with the observation that PAMs obtained from different pigs vary in their PRRSV-permissiveness, the results demonstrate that PAMs may spontaneously elaborate, or acquire through drug treatment, resistance to PRRSV infection. Additional research in the area of drug-mediated regulation of PRRSV permissiveness is needed to fully explore the significance of these observations.

IV. Introduction: Mechanisms regulating PRRSV infection are still poorly understood, and a better understanding of PRRSV infection is required to design antiviral drugs which might be of value to pork producers. Previous studies of PRRSV infection of MARC-145 cells have established that PRRSV has the ability to be highly cytotoxic to cultured cells, spreading by cell-to-cell transmission to essentially kill most cells within 3-5 days post-infection. It is of interest to study the behavior of PRRSV in pig macrophages since of course they are the most relevant cells for PRRS. Thus experiments were carried out to examine the pig macrophage response to PRRSV exposure as well as antiviral drug treatments that have been previously shown to have efficacy in cultured MARC-145 cells.

V. Objectives: The objectives were to examine PRRSV spread in cultured PAMs as well as the response to antiviral drugs. These objectives were based on preliminary data obtained from the study of PRRSV infection of MARC-145 cells. From this pilot project, it is hoped that a focused direction for future studies can be predicted.

VI. Materials & Methods: Porcine cells were obtained from individual 4-8 week-old pigs (about 5 for this project) by lung lavage and stored at –70 Cº until use. Aliquots of these cells were rapidly thawed and cultured overnight in DMEM containing 10% FBS. Non-adherent cells were removed and the adherent cells (designated porcine alveolar macrophages or PAMs) were inoculated with PRRSV (m.o.i. of about 0.5). For some experiments antiviral treatment of the cultured PAMs was carried out, using two distinct approaches: antiviral cytokine treatment (recombinant human IFN-gamma or AK-2); and antiviral quinolones (Plasmocin or nalidixic acid). Control antibiotics (erythromycin; penicillin; streptomycin) were also included in the experimental design. Drug dosages are indicated in the Results below. At various times post-infection, the PAMs were fixed in acetone and then probed with FITC-SDOW17, followed by

confocal microscopy. This approach allowed both quantitative and qualitative assessment of the cell response to PRRSV and antiviral drugs. Comparison of the response to virus infection and antiviral treatments was also made between PAMs and MARC-145 cells in these experiments.

VII. Results: Key findings of the project along with some data illustrations are listed below.

PRRSV was found to be autoregulated in PAMs, in contrast to the course of PRRSV infection in MARC-145 cells. Within two days post-infection, many cell cultures essentially recovered from PRRSV infection. There was some cell-to-cell spread of PRRSV in cultured PAMs, but at much lower levels than seen in cultured MARC-145 cells. Autoregulation of PRRSV was found to be associated with a soluble viral inhibitor (Fig.1 and Table 1 below), most likely a cytokine which inhibits PRRSV permissiveness (further studies are needed to confirm the mechanism). PAMs from different pigs also displayed variation in PRRSV permissiveness (data not shown).

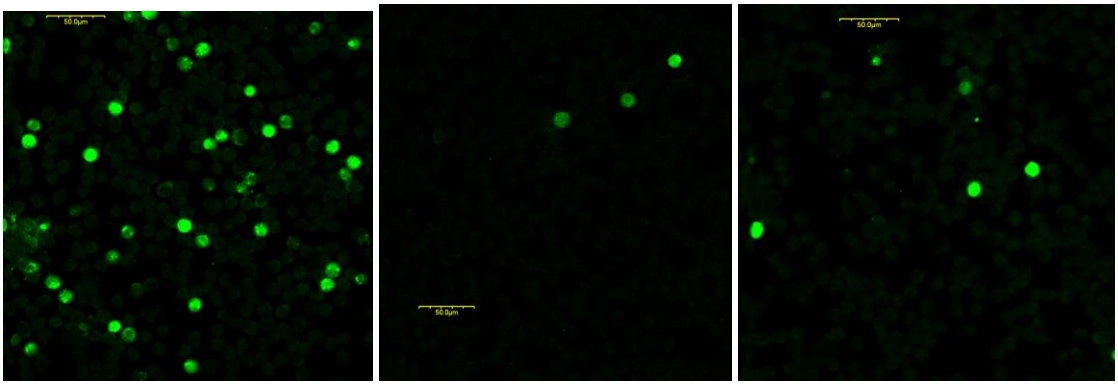

Figure 1. Illustration of autoregulation of PRRSV infection by PAMs. PRRSV stock was diluted in control MEM (A) or conditioned medium (B) from PAMs at 27 h p.i and used to inoculate naïve PAMs. The PAMs were then fixed at 17 h p.i. and stained with SDOW17. The results show ~90% inhibition of PRRSV infection by the conditioned medium (despite the presence of added virus due to the infection at 27 h p.i.). The donor culture was fixed at 44 h p.i. and stained for PRRSV Ag, to illustrate the autoregulation of PRRSV infection (C) by this time p.i.. (Cafruny et al. 2008; in preparation)

Table 1. Conditioned medium from PRRSV-infected (27 h p.i.) or uninfected (control) PAMs was used as the diluent for PRRSV, followed by inoculation of naïve PAM or MARC-145 target cells. Results are % of control at 17.5 h p.i..

Viral targets	Effect of PAM conditioned medium on PRRSV infection	
	27 h p.i with PRRSV	Control uninfected
PAMs	0% of control	74 % of control
MARC-145	0%	0%

These data show that the soluble inhibitor of PRRSV infection of PAMS is induced by PRRSV infection. The MARC-145 cell data suggest that PAMs also naturally produce a PRRSV inhibitor which MARC-145 cells are highly responsive to. (Alternatively, there may have been some contamination with PAMs which resulted in a xenogeneic response, but this possibility is considered unlikely.) Further studies are needed to resolve this possibility. (Cafruny et al., 2008; in preparation)

We reported previously that PRRSV infection of MARC-145 cells or PAMs was highly sensitive to 1 ug/ml antiviral cytokine treatment, which was capable of >90% inhibition of PRRSV permissiveness (Cafruny et al., 2006; and see Table 3 below). Also like the situation in MARC-145 cells, PRRSV infection of PAMs responded to antiviral quinolones (Table 2). Suboptimal drug combinations of quinolones and antiviral cytokines (drug synergy) were also effective in reducing PRRSV susceptibility of PAMs (data from PAMs and also MARC-145 cells shown in Table 3 below). In some cases, synergy was apparent even though suboptimal single drug exposure had no effect, since drug concentrations were below the threshold for efficacy.

Table 2. Drug responses of primary alveolar porcine macrophages. (from Cafruny et al., 2008 in press)

Drug treatment[1]		Expt.	Percent PRRSV-positive cells relative to control
Plasmocin	250 ug/ml	1	0
	25 ug/ml	2	0
	125 ug/ml	3	28
	125 ug/ml	4	38
	125 ug/ml	5	0
	125 ug/ml	6	9
	50 ug/ml	6	80
	25 ug/ml	6	76
Nalidixic acid	250 ug/ml	1	21
	250 ug/ml	2	18
	250 ug/ml	3	20
	250 ug/ml	4	0
	250 ug/ml	5	13
Erythromycin	125 ug/ml	1	122
	250 ug/ml	2	98
	125 ug/ml	2	104
	62 ug/ml	2	106

[1] PAMs were treated with the indicated drug doses, testing responses in individual cultures against matched control (no drug) cultures for each experiment.

Table 3. Potentiation of cytokine-mediated inhibition of PRRSV replication by nalidixic acid: drug synergy with sub-optimal drug concentrations (from Cafruny et al., 2008 In Press)

Expt.	Cells	Treatment/Pretreatment[1]	PRRSV response[2]
1.	PAMs (Treatment)	Nalidixic acid (25 ug/ml)	81 % of control
		AK-2 (125 ng/ml)	74 % of control
		AK-2 + Nalidixic acid	45 % of control
2.	PAMs (Pretreatment)	Nalidixic acid (25 ug/ml)	107 % of control
		AK-2 (125 ng/ml)	145 % of control
		AK-2 + Nalidixic acid	20 % of control
3.	PAMs (Pretreatment)	Nalidixic acid (25 ug/ml)	114 % of control
		IFN-γ (400 ng/ml)	103 % of control
		IFN-γ + Nalidixic acid	31% of control
4.	MARC-145 (Pretreatment)	Control	251 +/- 27 positive cells
		Nalidixic acid (25 ug/ml)	221 +/- 20 positive cells
		AK-2 (62 ng/ml)	38 +/- 6 positive cells
		AK-2 + Nalidixic acid	10 +/- 4 positive cells
5.	MARC-145 (Pretreatment)	Control	363 +/- 30 positive cells
		Nalidixic acid (25 ug/ml)	543 +/- 44 positive cells
		AK-2 (31 ng/ml)	166 +/- 42 positive cells
		AK-2 + Nalidixic acid	104 +/- 14 positive cells
6.	MARC-145 (Pretreatment)	Control	136 +/- 22 positive cells
		Nalidixic acid (25 ug/ml)	164 +/- 55 positive cells
		IFN-γ (400 ng/ml)	68 +/- 1 positive cells
		IFN-γ + Nalidixic acid	40 +/- 10 positive cells
7.	MARC-145 (Pretreatment)	Control	514 +/-17 positive cells
		Nalidixic acid (25 ug/ml)	570 +/-33 positive cells
		IFN-γ (200 ng/ml)	587 +/- 32 positive cells
		IFN-γ + Nalidixic acid	479 +/- 26 positive cells
8.	MARC-145 (Pretreatment)	Control	192 +/-2 positive cells
		Nalidixic acid (25 ug/ml)	188 +/-33 positive cells
		IFN-γ (400 ng/ml)	114 +/- 4 positive cells
		IFN-γ + Nalidixic acid	75 +/-2 positive cells

[1]Pretreatment consisted of 5 h incubation followed by removal of drugs and inoculation with PRRSV. For the treatment protocol, drugs were present throughout the virus exposure phase as well as during a one-h pretreatment period.

[2] PAM responses are reported as the percent infection relative to control cultures; MARC-145 cell responses are reported as mean total PRRSV-positive cells/culture +/- SD for duplicate wells.

Results from this project appear in a recently-accepted peer-reviewed publication; were presented at a national PRRSV conference; and contributed to an invention disclosure report:

Cafruny WA, Duman RG, Rowland RR, Nelson EA & Wong GHW. 2008. Antibiotic-mediated inhibition of porcine reproductive and respiratory syndrome virus (PRRSV) infection: a novel quinolone function which potentiates the antiviral cytokine response in MARC-145 cells and pig macrophages. Virology: Research & Treatment; In Press.

Presented at the 2007 International PRRSV Symposium, Chicago, Nov. 30-Dec.1:
Inhibition of PRRSV replication by quinolone-containing antibiotics. WA Cafruny, RG Duman, RR Rowland, EA Nelson, and GH Wong

Invention disclosure report 8-3-07:
Title: Quinolone treatment for infection with porcine reproductive and respiratory syndrome virus (PRRSV)

VIII. Discussion: These results demonstrate that pig macrophages are able to elaborate a natural resistance to PRRSV infection. Other PRRSV researchers working with PAMs may have also noticed variation in viral permissiveness of PAMs obtained from different pigs. We propose that PRRSV resistance may differ from pig-to-pig, likely due to different physiological status of individual pigs (for example the presence of other infections or different cytokine environments). Our results suggest that the natural resistance of pig macrophages to PRRSV is readily inducible in vitro, and limits virus spread between pig cells. Consistent with this hypothesis, antiviral drugs which elicit reduced cellular permissiveness to PRRSV replication are effective in suppressing PRRSV infection in vitro. Combination therapy with two distinct classes (cytokines and quinolones) of antiviral drugs (i.e. drug synergy) may be significant as an approach to reduce potential drug toxicity, which might be more prominent with single-drug treatment. Overall, these results have broad significance for the future development of anti-PRRS drug strategies, as well as an enhanced understanding of PRRSV regulation. The outcomes support the need for additional research to build on the results of this pilot project.